ORIGINAL ARTICLE

# The Characterization of an Adrenergic Signalling System Involved in the Encystment of the Ocular Pathogen *Acanthamoeba* spp.

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## ABSTRACT

The aim of this study was to identify and characterize the receptor system involved in controlling encystment in *Acanthamoeba* using specific agonists and antagonists and to examine whether endogenous stores of catecholamines are produced by the organism. *Acanthamoeba* trophozoites suspended in axenic growth medium were exposed to adrenoceptor agonists and antagonists to determine which compounds promoted or prevented encystment. Second, trophozoites were cultured in medium containing a catecholamine synthesis inhibitor to investigate the effect this had on natural encystment. Nonspecific adrenoceptor agonists including epinephrine, isoprotenerol, and the selective β1 adrenoceptor agonist dobutamine were found to cause > 90% encystment of *Acanthamoeba* trophozoites compared to < 30% with the controls. The selective β1 antagonist metoprolol was able to inhibit epinephrine mediated encystment by > 55%. Cultures of *Acanthamoeba* with the catecholamine synthesis inhibitor α-methyl-p-tyrosine significantly reduced the level of amoebic encystment compared to controls. In conclusion, *Acanthamoeba* appear to contain a functional adrenergic receptor system of unknown structure which is involved in initiating the encystment process that can be activated and blocked by β1 agonists and antagonists respectively. Furthermore, the presence of this receptor system in *Acanthamoeba* indicates that topical β adrenoceptor blockers may be effective adjunct therapy by reducing the transformation of trophozoites into the highly resistant cyst stage.

*ACANTHAMOEBA* is a common environmental free-living amoeba characterized by a life cycle of a feeding and replicating trophozoite which, in response to adversity, can transform into a highly resistant cyst stage (Byers et al. 1980; Cordingley et al. 1996). *Acanthamoeba* spp. are opportunistic pathogens of humans, notably causing keratitis in previously healthy persons. Contact lens wearers are most at risk from Acanthamoeba keratitis and account for some 90% of reported cases (Radford et al. 1998; Tu 2014).

Acanthamoeba keratitis is one of the most difficult ocular infections to manage successfully and can result in permanent blindness. Current medical therapy involves topical administration of a biguanide (either polyhexamethylene biguanide or chlorhexidine digluconate) in combination with a diamidine compound (propamidine isethionate or hexamidine diisethionate) (Lorenzo-Morales et al. 2015). Even with prompt diagnosis and aggressive medical therapy, the mean treatment period can be over 5 mo and in approximately 5% of cases the condition fails to respond to treatment (Dart et al. 2009). Many cases require a penetrating keratoplasty to either control the infection or resolve the damage caused by the organism and inflammatory response (Dart et al. 2009). The presence of dormant cysts in the corneal tissue can lead to recrudescence upon cessation of drug treatment causing infection of the corneal graft in up to 41% of patients after penetrating keratoplasty (Kitzmann et al. 2009).

A variety of chemical mediators including antibiotics, ethidium bromide, cyclic adenosine monophosphate (cAMP), taurine, and epinephrine have previously been shown to induce encystment (Akins and Byers 1980;

Raizada and Krishna Murti 1972; Srivastava and Shukla 1983b). In many eukaryotic cells cAMP-dependent signalling pathways play a critical role in regulating cell growth, metabolism, and differentiation and the levels of cAMP have been observed to increase in encysting *Acanthamoeba* (Abel et al. 2001; Raizada and Krishna Murti 1972). In mammalian systems, one mechanism for elevating intracellular levels of cAMP is through the action of the endogenous catecholamine epinephrine. In the liver this results in increased glycogen phosphorylase activity and the subsequent breakdown of the liver's glycogen stores (Riley and Haynes 1963). During encystment, glycogen metabolism is one of the key processes that has been observed in *Acanthamoeba* and it has been noted that the activation of adenylate cyclase, leading to polymer degradation, resembles the behaviour of the mammalian liver or muscle in its response to epinephrine (Verma and Murti 1976b; Weisman et al. 1970).

Furthermore, epinephrine and the synthetic catecholamine isoprotenerol have been shown to directly induce encystment in trophozoites of *A. culbertsoni* in the presence of magnesium (Srivastava and Shukla 1983b). Radio-labelled epinephrine has also been shown to be taken up by *Acanthamoeba* in a process blocked by the β adrenoceptor antagonist propranolol but not by the α antagonist phentolamine (Verma and Murti 1976a). Consequently, it has been suggested that the *Acanthamoeba* which interact with epinephrine, show some resemblance to the mammalian β adrenergic receptor in their sensitivity to the β adrenoceptor (βAR) antagonist propranolol (Verma and Murti 1976a).

Epinephrine has been reported to have been identified spectrophotometrically within *A. culbertsoni* in an unpublished observation (Verma et al. 1974) but conclusive evidence has not been published to date. Very little is known about exactly how *Acanthamoeba* senses and responds to its changing environment and the receptors and chemical pathways involved. However, previous studies with *Entamoeba invadens* have indicated that this organism contains a functional adrenergic signalling system through studies demonstrating the encystment promoting properties of catecholamines (Coppi et al. 2002) and through the identification of a G-protein-regulated adenylate cyclase that functions downstream from a adrenergic receptor (Frederick and Eichinger 2004).

Acanthamoeba keratitis is one of the most difficult ocular infections to treat and drugs including the diamidine compounds that are currently used in the treatment of Acanthamoeba keratitis have been reported to induce the encystment of *Acanthamoeba* at low concentrations by interfering with *Acanthamoeba* polyamine metabolism (Akins and Byers 1980; Ogbunude and Asiri 2001). Therefore, a compound which is able to prevent the transformation of trophozoites into the highly resistant cyst stage may prove to be useful tool for improving the treatment of this infection.

The aim of this study was to identify and characterize the receptor system involved in controlling encystment in *Acanthamoeba* using specific agonists and antagonists. This study will then determine if compounds related to epinephrine are synthesized and released by *Acanthamoeba*. To this end, this study has addressed the hypothesis that *Acanthamoeba* contains a functional adrenergic signalling system which enables them produce and store catecholamines that are released to initiate the encystment process via an interaction with adrenoceptors located on the surface of the trophozoite.

## MATERIALS AND METHODS

### *Acanthamoeba* strains and culture methods

*Acanthamoeba castellanii* (ATCC 30868) and *A. culbertsoni* (ATCC 30171) were obtained from LGC Standards (Teddington, UK). *Acanthamoeba polyphaga* (Ros) was isolated from a case of keratitis in 1991 (Kilvington et al. 1990). *Acanthamoeba* were cultured in 75 $cm^2$ tissue culture flasks (Nunc, Gloucester, UK) containing 30 ml of semi-defined axenic *Acanthamoeba* growth medium at 30 °C as previously described (Hughes et al. 2003). *Acanthamoeba castellanii* was selected for the encystment studies over *A. culbertsoni* and *A. polyphaga* because it has a more favourable encystment profile in growth medium supplemented with 50 mM $MgCl_2$ (Sigma, Gillingham, UK). Through personal observations, *A. culbertsoni* does not encyst in a nutrient encystment medium possibly through the presence of catabolite repressors that oppose encystment (Srivastava and Shukla 1983a). *Acanthamoeba polyphaga* too readily encysted making it difficult to tell what level of encystment was due to the exogenous catecholamines and what was due to natural encystment. *Acanthamoeba castellanii* was found to be in between these two extremes and gave intermediate levels of natural encystment in growth medium supplemented with 50 mM $MgCl_2$.

### Adrenoceptor agonist/antagonist studies

Encystment studies were performed in 50 ml polypropylene tubes containing $2.5 \times 10^5$/ml trophozoites in 5 ml *Acanthamoeba* axenic growth medium. All adrenoceptor agonists and antagonists were obtained from Sigma (Gillingham, UK). The required concentrations of $MgCl_2$ and α adrenoceptor (αAR) or β adrenoceptor (βAR) agonists and antagonists were subsequently added and the tubes were placed in an orbital shaking incubator at 30 °C and 120 rpm. Each day over a 7 d period the percentage encystment was recorded using a modified Fuchs Rosenthal haemocytometer (Hawksley, Lancing, UK).

### Inhibition of catecholamine synthesis

*Acanthamoeba polyphaga* was selected for the inhibition of catecholamine synthesis study over the other two strains as it readily encysted in trophozoite growth medium supplemented with 50 mM $MgCl_2$ in the absence of exogenous catecholamines. Two cultures of the *A. polyphaga* strain were set up and passaged three times

through either standard trophozoite growth medium or trophozoite growth medium supplemented with 10 mM of the catecholamine synthesis inhibitor α-methyl-p-tyrosine (AMPT) (Sigma). The two cultures were then transferred to trophozoite growth medium supplemented with 50 mM $MgCl_2$ to facilitate encystment.

### Data analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) of data from triplicate experiments on the InStat statistical software package (GraphPad, La Jolia, CA).

## RESULTS

### Adrenoceptor agonists

The results for the adrenoceptor agonist studies with *A. castellanii* are shown in Table 1. A low level of encystment of *Acanthamoeba* occurred in standard growth medium (GM) in the absence of $Mg^{2+}$ compared to 32% upon the addition of 50 mM $MgCl_2$ ($p > 0.05$). Encystment was further enhanced by the addition of epinephrine from 0.05, to 0.5, and up to 5 mM giving encystment of 56%, 72%, and 93% respectively ($p < 0.001$ for all relative to control). Isoproterenol induced approximately 90% encystment of *A. castellanii* ($p < 0.001$), whereas with norepinephrine only 45% encystment ($p < 0.001$). The selective $\beta AR_1$ agonist dobutamine gave encystment of 92% ($p < 0.001$) and the selective $\beta AR_2$ agonist salbutamol gave encystment of 39% at 10 μM ($p < 0.01$). Salbutamol could not be tested at higher concentrations as the compound caused extensive lysis of the *Acanthamoeba* trophozoites. The αAR agonist phenylephrine gave minimal encystment of approximately 10% at 100 μM ($p > 0.05$) and dibutyryl-cAMP, a lipophillic form of cyclic adenosine monophosphate which can cross intact membranes was also found to promote encystment of > 70% ($p < 0.001$).

**Table 1.** The effect of adrenoceptor agonists and dibutyryl cyclic adenosine monophosphate (cAMP) on the encystment of *Acanthamoeba castellanii*

| Encystment conditions | Agonist target | % Encystment (± SEM) |
|---|---|---|
| GM | None | 8.0 ± 0.7 |
| GM + $Mg^{2+}$ | None | 29.1 ± 2.9 |
| GM + $Mg^{2+}$ + Epinephrine (5 mM) | αAR/βAR | 93.2 ± 2.3 |
| GM + $Mg^{2+}$+ Epinephrine (0.5 mM) | αAR/βAR | 75.3 ± 3.5 |
| GM + $Mg^{2+}$ + Epinephrine (0.05 mM) | αAR/βAR | 56.0 ± 7.2 |
| GM + $Mg^{2+}$ + Isoprotenerol (1 mM) | αAR/βAR | 92.0 ± 2.3 |
| GM + $Mg^{2+}$ + Norepinephrine (5 mM) | αAR/βAR | 41.5 ± 4.0 |
| GM + $Mg^{2+}$ + Phenylephrine (100 μM) | αAR | 9.5 ± 1.7 |
| GM + $Mg^{2+}$ + Dobutamine (100 μM) | $\beta AR_1$ | 92.4 ± 2.0 |
| GM + $Mg^{2+}$ + Salbutamol (10 μM) | $\beta AR_2$ | 38.8 ± 3.8 |
| GM + $Mg^{2+}$ + Dibutyryl-cAMP (100 μM) | Increases cAMP | 72.6 ± 9.0 |

[a]The values reported are ± the standard error of the mean from triplicate experiments.
[b]GM (Growth media), $Mg^{2+}$ (50 mM $MgCl_2$), αAR/βAR (beta/alpha adrenoceptor).

### Adrenoceptor antagonists

The results for the adrenoceptor antagonist studies with *A. castellanii* are shown in Table 2, Epinephrine was added to the growth medium at a concentration of 500 μM along with 50 mM $MgCl_2$ and 500 μM of the antagonist to be tested. In the absence of antagonist, 0.5 mM epinephrine gave 72% encystment of *A. castellanii*. When the αAR antagonist phentolamine was added no decrease in the level of encystment was observed ($p > 0.05$). With the two nonselective βAR antagonists' alprenolol and propranolol the level of encystment decreased to 39% and 44% respectively ($p < 0.01$ for both). A small decrease of < 10% in encystment with the selective $\beta AR_2$ antagonist ICI-118,551 ($p > 0.05$) was observed in contrast with the > 55% increase in the level of encystment seen with the $\beta AR_1$ antagonist metoprolol ($p < 0.001$).

### Inhibiting catecholamine synthesis using AMPT

The results for the catecholamine synthesis inhibitor studies with *A. polyphaga* are shown in Table 3, *A. polyphaga* trophozoites grown in standard growth medium gave maximal encystment of 65% at 7 d compared to only 18% with the trophozoites grown in the presence of AMPT prior to encystment ($p < 0.001$).

## DISCUSSION

The resistance of *Acanthamoeba* cysts to most antimicrobial agents at concentrations tolerated by the cornea makes Acanthamoeba keratitis one of the most difficult

**Table 2.** The effect of adrenoceptor antagonists on the epinephrine mediated encystment of *Acanthamoeba castellanii*

| Encystment conditions | Antagonist target | % Encystment (± SEM) |
|---|---|---|
| GM + $Mg^{2+}$ + Epinephrine (500 μM) | None | 75.3 ± 3.5 |
| GM + $Mg^{2+}$ + Epinephrine (500 μM) + Phentolamine (500 μM) | αAR | 74.0 ± 6.4 |
| GM + $Mg^{2+}$ + Epinephrine (500 μM) + Alprenolol (500 μM) | βAR | 36.0 ± 3.1 |
| GM + $Mg^{2+}$ + Epinephrine (500 μM) + Propranolol (500 μM) | βAR | 40.7 ± 5.7 |
| GM + $Mg^{2+}$ + Epinephrine (500 μM) + Metoprolol (500 μM) | $\beta AR_1$ | 18.8 ± 2.0 |
| GM + $Mg^{2+}$ + Epinephrine (500 μM) + ICI 118,551 (500 μM) | $\beta AR_2$ | 59.7 ± 6.5 |

[a]The values reported are ± the standard error of the mean from triplicate experiments.
[b]GM (Growth media), $Mg^{2+}$ (50 mM $MgCl_2$), αAR/βAR (beta/alpha adrenoceptor).

**Table 3.** The effect of the catecholamine synthesis inhibitor α-methyl-p-tyrosine (AMPT) on the encystment of *Acanthamoeba polyphaga*

| | % Encystment (±SEM) | |
|---|---|---|
| Time | GM | GM + AMPT |
| Day 2 | 20.6 ± 0.2 | 8.0 ± 1.6 |
| Day 4 | 31.8 ± 1.8 | 9.4 ± 1.1 |
| Day 7 | 64.8 ± 3.9 | 18.2 ± 1.1 |

[a]The values reported are ± the standard error of the mean from triplicate experiments.
[b]GM (Growth media).

ocular infections to manage and requires intensive and prolonged medical therapy (Duguid et al. 1997; Elder et al. 1994; Marciano-Cabral and Cabral 2003). Gaining greater understanding of the mechanism behind the process by which the trophozoite transforms into the highly resistant stage will enable us to develop strategies for preventing encystation in vivo which will in turn reduce treatment time and prevent recrudescence of infection.

The nonspecific adrenoceptor agonists' epinephrine and isoprotenerol showed strong encystment promoting activity in *Acanthamoeba*. The maximal encystment obtained with norepinephrine another nonspecific adrenoceptor agonist was approximately 50% lower, suggesting it was only having partial agonist activity. When using the specific adrenoceptor agonists we found that αAR agonist phenylephrine had no encystment promoting activity, whereas selective $\beta AR_1$ agonist produced maximal encystment equivalent to epinephrine albeit at a 50 fold lower concentration. This result suggest that the type of adrenoceptor found in *Acanthamoeba* is of the $\beta AR_1$ subtype but the issue is confused by the observation that salbutamol a specific $\beta AR_2$ agonist exhibited partial agonist activity producing encystment at a level that was 50% of that seen with dobutamine. The exact reason for salbutamol and norepinephrine acting as partial agonists when we would expect salbutamol to have no effect, and norepinephrine to be equipotent with epinephrine is unclear. However, we must remember that adrenoceptor classification was developed in mammalian systems and we are trying to apply this categorization to a single celled amoeba. It is possible that we are looking an evolutionary primitive form of the receptor to that which we see in mammals and any structural differences may mean agonists behave differently to what is expected. Furthermore, the epinephrine concentrations used in this study were similar to those used in a previous study with *Entamoeba* spp. but these are significantly higher than those expected physiologically (Coppi et al. 2002). The difference in the concentrations required for activating the receptors suggests that there are some fundamental differences in the structure of the receptor compared to that found in mammalian systems and that the natural ligand is not epinephrine but could possibly be a metabolite of the catecholamines.

In the antagonist experiments our findings demonstrated that the αAR antagonist phentolamine had no effect on epinephrine-mediated encystment which supports the earlier observation that αAR agonists did not enhance the encystment process. The βAR antagonists alprenolol and propranolol both caused a reduction in the level of encystment of around 30% with the specific $\beta AR_1$ antagonist metoprolol causing biggest decrease of > 55%. The $\beta AR_2$ antagonist gave a < 10% reduction in the encystment levels which suggests that the $\beta AR_2$ adrenoceptor is not involved in the encystment process. Combined, the results indicate that selective $\beta AR_1$ agonists and antagonists can be used to promote and inhibit the encystment process. Furthermore, in an unpublished observation we have demonstrated that metoprolol can completely inhibit natural encystment triggered by Neff's defined encystment medium (Neff et al. 1964) in all three strains of *Acanthamoeba* used in this study.

These findings mirror that of a previous study with *E. invadens* which also demonstrated that $\beta AR_1$ agonists and antagonists can be used to promote and inhibit the encystment process. Using a radio-labelled nonselective βAR agonist [$^3$H]-CGP-12177, the authors were also able to show that nonlabelled βAR antagonists including metoprolol could block the binding of [$^3$H]-CGP-12177 and prevent encystment in a dose-dependent manner (Coppi et al. 2002). Other protozoa are believed to contain adrenergic signalling systems including the ciliate *Tetrahymena* through its response to αAR and βAR agonists and antagonists (Blum 1967).

Studies with dibutyryl-cAMP a nonhydrolysable form of cAMP which readily crosses cell membrane demonstrated that it too promoted encystment. This combined with the agonist and antagonist studies initially lead us to believe that the receptor was similar to the mammalian $\beta AR_1$ adrenoceptor. In the human body $\beta AR_1$ is known to be primarily coupled to the $G_s$ protein which activates adenylate cyclase leading to the intracellular accumulation of cAMP and the subsequent activation protein kinase A (Harmar et al. 2009). During normal amoebic growth intracellular cAMP levels are constant in the *Acanthamoeba* trophozoite but increase when the trophozoite encysts (Gessat and Jantzen 1974; Neff and Neff 1969). In mammalian systems cAMP is broken down to 5′ monophosphate by the enzyme phosphodiesterase (PDE) (Butcher and Sutherland 1962) and studies have shown that PDE inhibitors like theophylline can increase the level of encystment in *A. culbertsoni* (Verma et al. 1974).

However, although the results suggest the involvement of a $\beta AR_1$ receptor a search of the recently sequenced *Acanthamoeba* genome indicates that there are no sequences that share homology with known mammalian adrenergic receptor systems present. Therefore, further studies will be required to determine the structure of the receptor that the $\beta AR_1$ agonists and antagonists are interacting with. Interestingly the *Acanthamoeba* genome has revealed that they encode thirty-five G-protein-coupled receptors involved in cell signalling (Clarke et al. 2013) and so the authors believe that an as yet unidentified receptor is involved in adrenergic signalling in *Acanthamoeba*.

Magnesium Chloride ($MgCl_2$) was also present in the encystment medium and appears to play some role in mediating the encystment process, as when present there was a three fold increase in encystment compared to the control. One possibility for the encystment promoting activity of $Mg^{2+}$ lies in its ability to increase the fluidity of the lipid bilayer by interacting with negatively charged phospholipids leading to the modification in activity of membrane bound enzymes (Fuyu et al. 1983). Previous authors have demonstrated that βAR stimulation in cardiac and liver cells results an increase in levels of cAMP and a concomitant release of $Mg^{2+}$ (Romani et al. 1991). Probably one of the most significant processes that has been shown to occur during the encystment of *Acanthamoeba* is the breakdown of intracellular glycogens store (Weisman et al. 1970). In this process glycogen is degraded to glucose-1-phosphate by the enzyme glycogen phosphorylase (Heilmeyer 1991). The resulting glucose is then polymerized to form the cellulose cyst wall (Neff and Neff 1969). Divalent metal ions including $Mg^{2+}$ have been demonstrated to increase the affinity of phosphorylase kinase for glycogen phosphorylase resulting in enzyme activation (Xu et al. 1996). Therefore, this may explain how the presence of $Mg^{2+}$ in the medium promotes the encystment process by increasing the liberation of glucose from glycogen making more sugar available for cyst wall synthesis.

Synthesis of catecholamines like epinephrine occurs through a stepped synthesis pathway directly from the amino acid tyrosine or indirectly from phenylalanine. The rate limiting step in this pathway involves the conversion of tyrosine to DOPA by the enzyme tyrosine hydroxylase (Nagatsu et al. 1964). Assuming that *Acanthamoeba* have a catecholamine synthesis pathway, we incubated trophozoites with a catecholamine synthesis inhibitor to see if there was any effect on the encystment process. This study showed that 10 mM AMPT could competitively inhibit the enzyme tyrosine hydroxylase by competing with the free tyrosine in the medium (0.55 mM). This lead to a reduction in the encystment of *A. polyphaga* of approximately 40% compared to the control after 7 d in an encystment medium. Therefore, the results from this study indicate that *Acanthamoeba* spp. can synthesize and store catecholamines which are released to initiate encystment. This finding is supported by a previous study with the ciliate *Tyriformis* and the flagellate *Crithidia fasiculata* which showed radiolabelled $^{14}$C-Tyrosine, $^{14}$C-Phenylalanine amino acids became incorporated into the catecholamines in these organisms (Janakidevi et al. 1966).

In conclusion, the findings of this study suggest that *Acanthamoeba* spp. contain a functional adrenergic receptor system of unknown structure that can be activated and blocked by exogenous $\beta AR_1$ agonists and antagonists. In addition, the use of a catecholamine synthesis inhibitor this study demonstrates that *Acanthamoeba* synthesize and store catecholamines which are believed to be released to trigger the encystment process. *Acanthamoeba* have been shown to encyst in vivo during infection (Clarke et al. 2005), however, the exact reason for this unknown but it is possible they do this in response to catecholamines present in the patients circulatory system or contained within the lachrymal fluid.

The encysted form of *Acanthamoeba* is highly resistant to kill using topical antimicrobial agents and so it may be possible to use topical βAR antagonists as an adjunct therapy in Acanthamoeba keratitis alongside the current drug regime to reduce encystment of amoebae during treatment. Preventing encystment could potentially enhance treatment as the trophozoite is more susceptible to kill using topical antimicrobial agents. It is hoped that this approach could bring down the mean treatment time and prevent recrudescence of infection. Topical βAR antagonists are well tolerated in the eye and are currently used in the management of glaucoma (Ong et al. 2005).

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